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Nomenclature and functions of RNA-directed RNA polymerases

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There is little relationship between eukaryotic RNA-directed RNA polymerases (RDRs), viral RNA-dependent RNA polymerases (RdRps) and DNA-dependent RNA polymerases, indicating that RDRs evolved as an independent class of enzymes early in evolution. In fungi, plants and several animal systems, RDRs play a key role in RNA-mediated gene silencing [post-transcriptional gene silencing (PTGS) in plants and RNA interference (RNAi) in non-plants] and are indispensable for heterochromatin formation, at least, in *Schizosaccharomyces pombe* and plants. Recent findings indicate that PTGS, RNAi and heterochromatin formation not only function as host defence mechanisms against invading nucleic acids but are also involved in natural gene regulation. RDRs are required for these processes, initiating a broad interest in this enzyme class.

Identification of plant RNA-directed RNA polymerases

The activity of a plant RNA-directed RNA polymerase (RDR) was detected ~35 years ago in a search for enzymes that catalyse the replication of plant RNA viruses [1]. Upon infection with a virus, RDR activity was elevated, pointing to the misleading conclusion that RDRs are involved in virus replication. It turns out that virus genomes are not amplified by plant RDRs but by virus-encoded RNA-dependent RNA polymerases (RdRps) [2,3]. In plants, six *RDRs* (*RDR1–RDR6*) are expressed (see below) but *RDR2–RDR6* are expressed poorly. Viruses produce their own RdRp; therefore, any attempt to isolate plant RDRs from virus-infected material suffers from the difficulties in distinguishing between the plant and viral RNA polymerase activities [4]. *RDR1* is induced upon virus [2,3] and viroid infection [4] and by salicylic acid treatment [5]. Viroids do not code for proteins and their RNA/RNA replication is entirely dependent on host enzymes [6]. Thus, RNA polymerase activity that is detectable in viroid-infected plants must exclusively originate from host enzymes. Winfried Schiebel and co-workers took advantage of a virus replicase-free system and used viroid-infected tomato plants as a source to isolate the first plant RDR, now termed LeRDR1 [7,8]. The physicochemical [7] and *in vitro* catalytic [9] properties of the LeRDR1 were characterized (Box 1). In addition, the *LeRDR1* cDNA was isolated [8]. However, the biological function of the LeRDR1 remained elusive and could not be associated with virus and viroid replication.

Nomenclature of RNA-directed RNA polymerases

In view of the considerable advances in characterizing specific functions of RNA-directed RNA polymerases, the current nomenclature is outdated – the main purpose of this article is to propose an updated consistent terminology for this class of enzymes. Initial studies on an enzyme that transcribed RNA from RNA templates were published in 1963 [10,11]. This enzyme, which was termed RNA-dependent RNA polymerase, was encoded by a phage and not by a plant. Its biological function is to replicate the phage genome. Further characterization of virus genome-replicating enzymes led to the identification of numerous virus-encoded RdRps [12]. The first report on eukaryotic RNA polymerase activity appeared in 1971 [1]. The corresponding enzyme was isolated from Chinese cabbage and was analogous to the phage and viral enzymes termed RNA-dependent RNA polymerase. A series of studies on RNA polymerase activity in different plant species was subsequently published. Eventually, in 1981, all enzymes that catalyse RNA-template-directed extension of the 3′-end of an RNA strand by one nucleotide at a time were annotated in the IUBMB enzyme nomenclature database (http://www.chem.qmw.ac.uk/iubmb/enzyme) as RNA-directed RNA polymerases (EC 2.7.7.48). However, to date, the terms RNA-dependent RNA polymerase and RNA-directed RNA polymerase, as well as the associated acronyms RdRp and RdRP, are similarly used for viral replicases and host enzymes.

The abbreviation for plant *RdRP* genes was recently changed to *RDR* [13]. In the *Arabidopsis thaliana* genome, six sequences were identified displaying significant homology to *LeRDR1* [14]. They were specified as *AtRDR1–AtRDR6*. However, based on phylogenetic analysis of all identified RDRs (see Supplementary material), we suggest renaming the *Arabidopsis* genes as *AtRDR1*, *AtRDR2*, *AtRDR3a*, *AtRDR3b*, *AtRDR3c* and *AtRDR6* (see below). Plant orthologues of the tomato *RDR* founder sequence [8] are now termed *RDR1*, and orthologues associated with *de novo* methylation and heterochromatin formation are known as *RDR2*. The name of the *Arabidopsis SDE1/SGS-2* homologues that are involved in PTGS will remain unchanged (*RDR6*) and, based on the unique DFDGD amino acid sequence motif of their catalytic domain (see Supplementary material), *RDR3–RDR5* will be renamed *RDR3a–RDR3c*. In

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Box 1. Biochemical properties of eukaryotic RNA-directed RNA polymerases

To date, there are only three reports on the biochemical characterization of plant purified or recombinant RDR activity [9,27,41]. Winfried Schiebel and co-workers [9] showed that the tomato RDR1 catalyses RNA synthesis *in vitro*. Using a CpG dinucleotide as a primer, the LeRDR1 synthesized full-length complementary RNA from 13-nt ssRNA as well as from 14-nt ssDNA templates. They further demonstrated that unprimed RNA transcription could be initiated at the 3′ terminus of ssRNA and ssDNA templates. In addition to the transcription capacity, the LeRDR1 possesses a terminal transferase activity that preferentially adds adenosine or guanosine residues to the 3′ ends of the RDR1 products. dsDNA did not serve as a substrate for any of the LeRDR1 activities.

Transitive silencing in plants requires the production of secondary siRNAs, which, in turn, depends on a functional RDR6. In plants, silencing spreads in 5′–3′ and 3′–5′ directions [36,39,40], although a preference for the 5′–3′ direction was observed [40]. RNA synthesis proceeds only in the 5′–3′ direction. Thus, the preferential 5′–3′ spreading of silencing implies that RDR6 mainly acts in a primer-independent manner. However, siRNAs might mark templates for copy RNA synthesis in that the AGO1-bound siRNAs serve as bridging molecules to tether the RDR6 to the 3′ end of the target RNA.

Similarly, a recombinant QDE-1 catalysed both un-primed and primer-dependent RNA synthesis *in vitro* [27]. Compared with the unprimed function, primer extension was inefficient and probably not the main function of QDE-1. Interestingly, most known polymerases rely on a *de novo* initiation mechanism [74,75] with only a few exceptions where short oligonucleotides or proteins are used as primers. The recombinant QDE-1 produced full-length dsRNA from ssRNA templates, and initiated *de novo* RNA synthesis at internal template sites more efficiently, which generated 9–21-nt complementary RNA molecules scattered along the entire template. Thus, it is reasonable to speculate that the 21-nt products are directly loaded onto an Argonaute protein to guide a RISC-like nuclease complex to its target.

In wheat germ extracts, RDR activities resulted in the copying of exogenous ssRNA into complementary RNA of approximately template length [41]. These data confirmed the previous results indicating that at least some of the plant RDRs do not require primers to transcribe ssRNA. However, ssRNA was not added in physiological concentrations to the wheat germ extracts. In summary, the biochemical analysis data are consistent with our PTGS model in that RDRs appear to generate complementary RNA from ssRNA templates by un-primed and primed mechanisms. In the context of our model, it will be interesting to elucidate whether plant RDRs are able to use mature mRNAs as a template or whether only abRNAs are copied.

addition, the taxon abbreviation of the species could be prefixed. For example, the tomato *RDR1* gene could be named *LeRDR1*, indicating its origin from *Lycopersicon esculentum*. Accordingly, the present terminology for *AtRdRP1* (*Arabidopsis*) [14], *NtRdRP1* (*Nicotiana tabacum*) [5] and *MtRdRP1* (*Medicago truncatula*) [15] genes could then be *AtRDR1*, *NtRDR1* and *MtRDR1*, respectively.

The nomenclature became confusing when genetic analysis of mutants presented the first experimental evidence for RDR involvement in gene silencing. The identified genes were named and numbered according to the phenotypic appearance of the corresponding mutants. For *Neurospora crassa*, transgene-induced gene silencing is known as quelling. An *RDR* orthologue that was isolated from a genetic screen (in which mutants were impaired in gene silencing) was consequently specified as 'quelling-defective gene 1' (*QDE-1*) [16]. The *Arabidopsis AtRDR6* gene was initially named in accordance with gene silencing-deficient mutants. Tamas Dalmay and co-workers [17] reported that the 'silencing-defective gene 1' (*SDE1*) shared substantial homology with *LeRDR1*. In parallel, Philippe Mourrain and co-workers [18] found that the 'suppressor of silencing gene 2' (*SGS-2*) corresponded to the same gene. Mutations of the *Caenorhabditis elegans* 'enhancer of Glp-One gene' (*EGO-1*) exhibited defects in germ-line development [19]. Later, it became evident that the *EGO-1* product is a component of the RNAi machinery [20]. Amino acid sequence alignment revealed a close relationship between EGO-1, LeRDR1 and QDE-1. Three additional protein sequences were retrieved from the *C. elegans* database that showed substantial homology to the known RDRs. They were designated the RNA-dependent RNA polymerase family genes 1–3 (*RRF-1*, *RRF-2*, *RRF-3*) [20,21]. *Dictyostelium discoideum* expresses three *RDR* genes – *RrpA*, *RrpB*, and *DosA*, which was recently renamed *RrpC* (http://dictybase.org). The *D. discoideum* RDRs differ from other eukaryotic RDRs in that they contain an N-terminal helicase domain with high homology to the helicase domain of a Dicer enzyme expressed in *C. elegans*. The two dicer orthologues of *D. discoideum* do not contain a helicase motif, and therefore, domain swapping between these enzymes has been suggested [22]. Only a single *RDR* orthologue, *Rdp1*, is expressed in *Schizosaccharomyces pombe*. This enzyme is essential for both RNA-mediated heterochromatin formation and RNAi [23,24]. The current abbreviations of the eukaryotic RNA-directed RNA polymerases are summarized in Tables 1 and 2.

To specify RNA-directed RNA polymerases more precisely, we suggest that the abbreviations RDR for eukaryotic RNA-directed RNA polymerases and RdRp for viral RNA-dependent RNA polymerases should be adhered to. In addition, a species abbreviation can be prefixed and a number for each homologue should be suffixed. However, there are no common rules to indicate the systematic origin of a gene or its product. Thus, the organism from which the gene or protein was isolated needs to be indicated to complete the nomenclature. Consistent with this terminology, the eukaryotic RNA-directed RNA polymerases should be renamed as indicated in Tables 1 and 2.

Apart from plants, RDRs cannot be classified according to their function or to their conserved amino acid sequence motifs. For example in *S. pombe*, the Rdp1 is associated with both RNAi and RNA-mediated heterochromatin formation (nuclear RNAi) [24]. This is in contrast to plants, where the RDR2 and RDR6 are required for nuclear RNAi and PTGS, respectively (see below). Thus, a name that reflected the *S. pombe* enzyme functions needs to contain two suffixed numbers and could be SpRDR2/6. Our multiple sequence alignment using the ClustalW program [25] (http://www.ebi.ac.uk/clustalw, see Supplementary material) revealed that the putative catalytic domain containing the DLDGD motif [26] is highly conserved among all identified RDRs (Box 2). However, within this motif, the lysine appeared to be variable. For example,

Table 1. Putative plant RNA-directed RNA polymerases identified by data base searches[a]

Old abbreviation	Organism (plants)	Putative function	New abbreviation	Accession no.
LeRdRP (cDNA)	*Lycopersicon esculentum*	Control of virus accumulation	LeRDR1	Y10403
NtRdRP1 (cDNA)	*Nicotiana tabacum*	Control of virus accumulation	NtRDR1	AJ011576
NbRdRP1m (cDNA)	*Nicotiana benthamiana*	NK (3′ end truncated RDR1)	NbRDR1	AY574374
RdRP-like (genomic DNA)	*Solanum tuberosum*	NK	StRDR1a	AC151802
RdRP-like (genomic DNA)	*Solanum tuberosum*	NK (3′ end truncated RDR1)	StRDR1b	AY730334
RdRP-like (genomic DNA)	*Solanum tuberosum*	NK (5′ and 3′ end truncated RDR1)	StRDR1c	AY730337
RdRP-like (genomic DNA)	*Solanum demissum*	NK	SdRDR1a	AC149287
RdRP-like (genomic DNA)	*Solanum demissum*	NK (3′ end truncated RDR1)	SdRDR1b	AC149291
RdRP-like (genomic DNA)	*Solanum demissum*	NK (5′ end extended RDR1 containing internal and 3′ end deletions)	SdRDR1c	AC144791
RdRP-like (genomic DNA)	*Solanum demissum*	NK (5′ end truncated RDR1)	SdRDR1d	AC149288
RdRP (partial genomic DNA)	*Petunia hybrida*	NK	PhRDR1	AJ011979
AtRdRP1 (RDR1) (cDNA)	*Arabidopsis thaliana*	Control of virus accumulation	AtRDR1	AC006917
RdRP1 (cDNA)	*Hordeum vulgare*	NK	HvRDR1a	AY500822
RdRP2 (cDNA)	*Hordeum vulgare*	NK	HvRDR1b	AY500821
RdRP-like (genomic DNA)	*Oryza sativa*	NK	OsRDR1	AP008208
RdRP-like (partial cDNA)	*Zea mays*	NK	ZmRDR1	AY103827
RdRP (partial cDNA)	*Triticum aestivum*	NK	TaRDR1	AJ011978
LeRdRP2 (cDNA)	*Lycopersicon esculentum*	NK	LeRDR2	Unpublished[b]
RdRP2 (cDNA)	*Nicotiana benthamiana*	NK	NbRDR2	AY722009
RDR2 (cDNA)	*Arabidopsis thaliana*	*De novo* methylation, heterochromatin formation	AtRDR2	AF080120
RdRP-like (cDNA)	*Oryza sativa*	NK	OsRDR2	AL606653
NtRDR3 (cDNA)	*Nicotiana tabacum*	NK	NtRDR6	Unpublished[b]
RdRP (cDNA)	*Nicotiana benthamiana*	NK	NbRDR6	AY722008
RDR6 (SDE1/SGS-2) (cDNA)	*Arabidopsis thaliana*	RNAi (PTGS) (initiation, maintenance biogenesis of tasiRNAs)	AtRDR6	AF268093
RdRP-like (genomic DNA)	*Medicago truncatula*	NK	MtRDR6	AC149808
RdRP-like (cDNA)	*Oryza sativa*	NK	OsRDR6	AP004357
AtRDR3 (genomic DNA)	*Arabidopsis thaliana*	NK (contains a DFDGD instead of the DLDGD signature)	AtRDR3a	At2g19910
AtRDR4 (genomic DNA)	*Arabidopsis thaliana*	NK (contains a DFDGD instead of the DLDGD signature)	AtRDR3b	At2g19920
AtRDR5 (genomic DNA)	*Arabidopsis thaliana*	NK (contains a DFDGD instead of the DLDGD signature)	AtRDR3c	At2g19930
RdRP-like (cDNA)	*Oryza sativa*	NK (contains a DFDGD instead of the DLDGD signature)	OsRDR3a	NM_188258
RdRP-like (cDNA)	*Oryza sativa*	NK (contains a DFDGD instead of the DLDGD signature)	OsRDR3b	NM_188259

Abbreviation: NK, not known.
[a]See Supplementary material.
[b]M. Wassenegger, unpublished data, see Supplementary material.

in *N. crassa* QDE-1, the lysine is replaced by a tyrosine. A recombinant QDE-1 exhibits RNA polymerase activity *in vitro* [27], and endogenous QDE-1 is essential for quelling, which might indicate its close functional relationship to plant RDR6, which is indispensable for S-PTGS (see below). Thus, QDE-1 should be renamed NcRDR6. There are two additional categories of RDRs that carry lysine substitutions: one category with a methionine and a second with a phenylalanine substitution (see Supplementary material). The first group comprises three orthologues from different but closely related fungi and the second is represented by three *Arabidopsis* and two *Oryza sativa* orthologues. The biological function of these proteins is not known. The plant RDRs containing the DFDGD motif all cluster in a phylogenetic group (see Supplementary material) and, because of their high homology, they should be specified as *AtRDR3a–AtRDR3c* and *OsRDR3a and OsRDR3b*, respectively, rather than as *AtRDR3– AtRDR5*, which is currently the case for the *Arabidopsis* genes. To date, only DLDGD-containing RDRs have been identified in plants other than *Arabidopsis* and *O. sativa*.

Based on their function and the extensive homology between the *C. elegans RRF-1* and *RRF-2* and between the *D. discoideum rrpA* and *rrpB* orthologues, these genes should be termed *CeRDR6a and CeRDR6b*, and *DdRDR6a* and *DdRDR6b*, respectively. It is difficult to classify RDRs according to their function in non-plant organisms, but, if known, the RDR function could be indicated by the suffixed number. The requirement of RRF-1 and rrpA for RNAi [21,22] suggests a relationship between these enzymes and plant RDR6. Thus, RRF-1 and RRF-2 and rrpA and rrpB might be specified as CeRDR6a and CeRDR6b, and DdRDR6a and DdRDR6b, respectively. *C. elegans* EGO-1 resembles *S. pombe* Rdp1 (SpRDR2/6) in that this enzyme is associated with RNAi in germline cells [20] and with heterochromatin assembly during meiosis [28]. In accordance with the suggested updated nomenclature, EGO-1 should be renamed CeRDR2/6.

Table 2. Putative non-plant RNA-directed RNA polymerases identified by data base searches[a]

Old abbreviation	Organism (animals)	Putative function	New abbreviation	Accession no.
RdRP-like (genomic DNA)	*Branchiostoma floridae*	NK	BfRDR1	AF537961
RRF-3	*Caenorhabditis elegans*	Reduction of RNAi sensitivity	CeRDR1	NM_063312
EGO-1	*Caenorhabditis elegans*	RNAi (germline)	CeRDR2/6	NM_059731
RRF-1	*Caenorhabditis elegans*	RNAi (somatic)	CeRDR6a	NM_059730
RRF-2	*Caenorhabditis elegans*	NK	CeRDR6b	NM_060656
	Organism (fungi)			
RDP-1	*Schizosaccharomyces pombe*	RNAi, heterochromatin formation	SpRDR2/6	Z98533
RDP-1	*Diaporthe ambigua*	NK (contains a DMDGD instead of the DLDGD signature)	DaRDR2	AY049072
RDP-1	*Diaporthe perjuncta*	NK (contains a DMDGD instead of the DLDGD signature)	DpRDR2	AF468822
RDP-1	*Phomopsis* sp. *CMW 5588*	NK (contains a DMDGD instead of the DLDGD signature)	P.spRDR2	AF443073
Hypothetical protein	*Gibberella zeae PH-1*	NK	GzRDR1	XM_381758
Hypothetical protein	*Gibberella zeae PH-1*	NK	GzRDR2	XM_388892
Hypothetical protein	*Gibberella zeae PH-1*	NK (contains a DYDGD instead of the DLDGD signature)	GzRDR6a	XM_384795
Hypothetical protein	*Gibberella zeae PH-1*	NK (contains a DYDGD instead of the DLDGD signature)	GzRDR6b	XM_386680
RdRP-like (Sad-1)	*Aspergillus fumigatus*	NK	AfRDR2	XM_749834
RdRP	*Aspergillus fumigatus*	NK (contains a DYDGD instead of the DLDGD signature)	AfRDR6	XM_748603
Hypothetical protein	*Aspergillus nidulans*	NK	AnRDR1	XM_655229
Hypothetical protein	*Aspergillus nidulans*	NK	AnRDR2	XM_657302
RdRP-like	*Neurospora crassa*	NK	NcRDR1	BX284762
RdRP-like (Sad-1)	*Neurospora crassa*	Suppressor of ascus dominance	NcRDR2	XM_329367
QDE-1	*Neurospora crassa*	RNAi (quelling) (contains a DYDGD instead of the DLDGD signature)	NcRDR6	AJ133528
Hypothetical protein	*Magnaporthe grisea*	NK	MgRDR1	XM_369259
Hypothetical protein	*Magnaporthe grisea*	NK	MgRDR2	XM_366672
rrpC	*Dictyostelium discoideum*	Antisense RNA production (W. Nellen, personal communication)	DdRDR1	XM_635698
rrpA	*Dictyostelium discoideum*	RNAi	DdRDR6a	XM_631001
rrpB	*Dictyostelium discoideum*	NK	DdRDR6b	XM_630171
RdRP-like	*Entamoeba histolytica*	NK	EhRDR1	XM_646217

Abbreviation: NK, not known.
[a]See Supplementary material.

Biological functions of eukaryotic RDRs

Post-transcriptional and virus-induced gene silencing in plants

The discovery of PTGS [29,30] and RNA-mediated virus resistance [31] in plants provided the first clues about the role of RDRs. PTGS was occasionally observed in plant lines carrying multiple copies of transgene constructs. In these lines, a dramatic decrease in the steady-state mRNA level was detected. Moreover, if the transgene shared homology with an endogene, expression of both genes was suppressed. This co-suppression phenomenon was post-transcriptional and based on a mechanism that we know today as an RNA-mediated plant surveillance mechanism. The same mechanism appeared to become activated upon virus infection of plants that expressed a transgene sharing homology with the infecting virus [32]. In 1993, John A. Lindbo and co-workers [31] suggested a mechanism in which an RDR copies the transgene RNA into small complementary RNAs. These small antisense RNAs would then hybridize with target RNAs, rendering them non-functional, and the partially double-stranded RNA (dsRNA) molecules would then be degraded by RNases that specifically recognize dsRNA. Lindbo's model was the first description of PTGS.

Involvement of RDRs in initiation and maintenance of PTGS and VIGS

PTGS and RNA-mediated virus resistance are closely related mechanisms. PTGS is induced by a dsRNA trigger that originates from an endogenous source, whereas RNA-mediated virus resistance is related to virus-induced gene silencing (VIGS). VIGS is initiated by dsRNA that is derived from the replicating virus, representing an exogenous source of trigger RNAs. In the case of PTGS, the dsRNA trigger either originates from primary transcription of a transgene that is rearranged during the genome-integration process as an inverted repeat (IR) or from artificial primary transcripts (abRNAs) of a sense transgene. abRNAs are assumed to accumulate over a certain threshold during high sense transgene expression. We suggest here that abRNA molecules preferentially serve as a template for the plant RDR6. Recent studies have indicated that mRNAs lacking a cap structure become exposed to an RDR [33]. However, non-polyadenylated mRNAs might also be considered as aberrant and might serve as efficient RDR substrates as well. In any case, extensive primary transcription of transgenes would lead to an accumulation of abRNAs that are transcribed into dsRNA by RDR6. This hypothesis was substantiated by the finding that in *Arabidopsis*, sense transgene-mediated

Box 2. Evolution and origin of RNA polymerases

The broad representation of RDRs in eukaryotes, including *Schizosaccharomyces pombe*, suggests that they originate in the genome of the common ancestor of all modern eukaryotes. However, the function of these enzymes has been subsequently lost in several eukaryotic lineages, for example, mammals. RDRs and DNA-dependent RNA polymerases (DDRPs) share a homologous catalytic core, the so-called double-psi-β-barrel (DPBB) [76]. In spite of the differences between template-dependent and template-independent polymerases, in all known cases the catalytic activity maps to a single polypeptide. It contains the signature motif DxDGD, which is a characteristic metal-chelating active site [77–79]. Typically this active site is composed of acidic or polar amino acid residues that coordinate divalent metal cations, in most cases Mg^{2+}. The metal cations direct a 5′ nucleoside triphosphate to form a phosphoester bond with the 3′ hydroxyl group of the preceding nucleotide, with the elimination of pyrophosphate [78]. The primordial RDR, which consisted primarily of the DPBB domain, might have evolved from a common ancestor to the DDRP at an early stage of evolution. It is conceivable that the ultimate ancestor of RNA polymerases was a RNA-binding DPBB domain that functioned as a co-factor for a polymerase ribozyme. Replacement of the ribozyme might have occurred when the DPBB acquired key residues that were required for protein-based polymerase activity [76].

The origin of other conserved motifs within the RDRs, and accordingly the evolutionary scenario for RDRs, remains less clear. It is conceivable that eukaryotic RDRs and viral RdRps are (unrelated) vestiges of an ancient DNA world tamed by DNA-based organisms or surviving in selfish genetic elements such as viruses and bacteriophages.

PTGS (S-PTGS) required AtRDR6 activity whereas IR transgene-mediated silencing (IR-PTGS) and RNA virus-induced gene silencing occurred in AtRDR6-deficient plants [34]. dsRNA is processed into 21-bp small interfering RNAs (siRNAs) by one of the Dicer-like (DCL) RNases III, predominantly by DCL4 [34,35]. As single strands, these siRNAs are bound to AGO1, a member of the Argonaute protein family. The AGO1-bound siRNA hybridizes with complementary RNA, thereby initiating cleavage of the target.

In addition to the initiation step, efficient PTGS requires a maintenance step that is based on RDR6-mediated amplification of the dsRNA trigger (primary dsRNA) or on generating secondary siRNAs. The PTGS maintenance mechanism was identified in plants in which silencing was locally initiated. Agroinfiltration with transgene constructs or infection with movement-deficient viruses, both resulted in localized production of dsRNA and in localized initiation of silencing [36,37]. In these types of experiments, silencing was found to spread from the cells, into which the primary dsRNA-producing construct was introduced ('silencing inducer cells'), into a constant number of surrounding cells (13 ± 2) ('siRNA receiver cells') [35,38]. Christophe Himber and co-workers [38] suggested that the 21-nt siRNAs that are produced in the 'silencing inducer cells' move from cell-to-cell via plasmodesmata channels into neighbouring 'siRNA receiver cells' where they target homologous RNA for cleavage (Figure 1). siRNA spreading was limited when an endogene was targeted, which suggests progressive dilution of the siRNAs and/or a highly controlled mechanism of siRNA propagation. In contrast to this limited cell-to-cell movement of silencing, extensive cell-to-cell movement of silencing occurred upon transgene targeting. This type of spreading of silencing is based on the production of secondary dsRNA. Importantly, in *C. elegans* and in plants, dsRNA synthesis proceeds from the siRNA/template binding sites into flanking sequences (transitive silencing) [21,36,37,39,40]. In the first step, and similar to limited spreading, the siRNAs move from the 'silencing inducer cells' into surrounding cells, thereby initiating target RNA cleavage. In the second maintenance step, transcripts of the transgene would not only become a target for degradation, but would also serve as a template for the RDR6 [Figure 1, abRNA (low)]. This step might include priming by siRNAs given that this has been detected in *C. elegans* [21]. However, in plants and *N. crassa*, RDR-mediated production of secondary dsRNA appears to also function in a primer-independent manner [27,40,41]. The secondary dsRNA is processed into secondary siRNAs that bind to AGO1. Thus, a 'siRNA receiver cell' becomes a 'silencing inducer cell' and the secondary siRNAs could move over a further distance of 11–15 cells to re-initiate the same process, which finally would lead to extensive cell-to-cell movement of silencing (Figure 1). Extensive cell-to-cell movement of the 21-nt siRNAs would then proceed until physiological effects (e.g. sink–source transition), morphological structures (e.g. veins) or the unavailability of the recently detected silencing movement-deficient proteins (SMD1, SMD2 and SMD3) [35] impair further 21-nt siRNA movement or function.

At present, it is not clear why transgene silencing is associated with secondary dsRNA production and transitive silencing whereas endogene silencing is generally not associated with either process [40,42–44]. We suggest that only primary transcription of transgenes involves high accumulation of abRNA and that RDR6 predominantly uses abRNAs as templates (Figure 1). Transgenes usually do not contain introns and, therefore, are not processed by the spliceosome. During the splicing process, aberrant endogenous transcripts might be efficiently eliminated before being exported into the cytoplasm. By contrast, transgene transcription does not involve the spliceosome, leading to accumulation of transgene-derived abRNA in the cytoplasm. In support of this hypothesis, transgene constructs that contained intron-less endogenous sequences triggered PTGS and extensive cell-to-cell movement of silencing [29,30,45,46]. Thus, endogenous sequences appear to resemble transgenes when transformed into the plant genome using state-of-the-art expression cassettes. These findings indicate that neither the sequence context, for example, C/G content, nor the level of transcription could account for the capacity to maintain PTGS.

As an alternative to splicosome-mediated elimination of abRNA, a mechanism in which abRNA production is controlled by the transcription machinery could be considered. State-of-the-art transgene expression cassettes usually contain viral- or *Agrobacterium tumefaciens*-related regulatory elements, such as the cauliflower mosaic virus 35S promoter, the nopaline synthase promoter and nopaline polyadenylation signal sequences. These elements can differ from plant regulatory elements

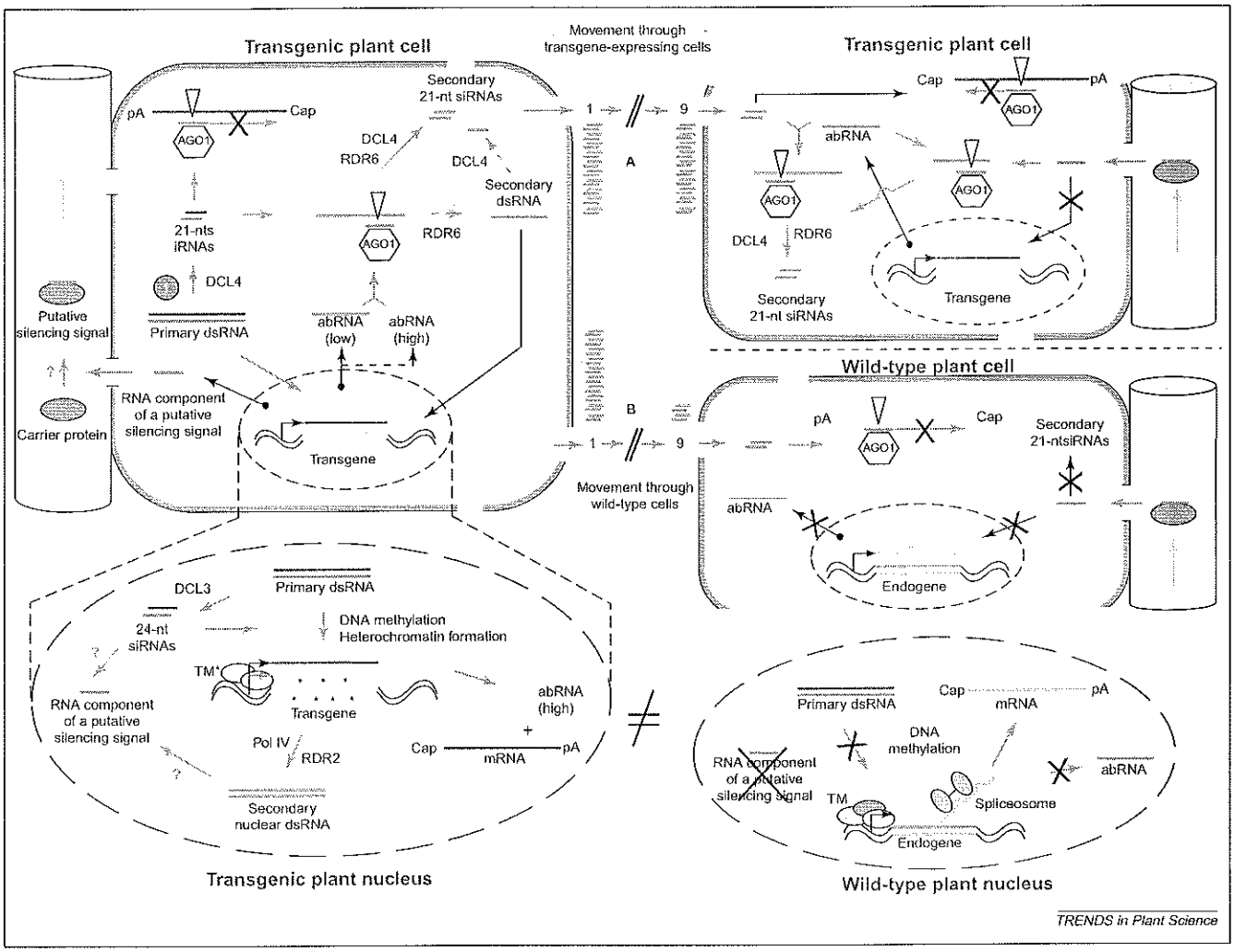

Figure 1. Model of the plant post-transcriptional gene silencing (PTGS) mechanism with a focus on the involvement of RNA-directed RNA polymerases (RDRs). Primary dsRNA initiates PTGS (indicated by '1' highlighted in red circle) and most of it is processed into 21-nt siRNAs by DCL4. The 21-nt siRNAs are loaded onto AGO1 to target complementary mRNA for cleavage. Targeting of mature mRNA would not recruit RDR6 activity. Thus, mRNA cleavage does not contribute to maintaining PTGS. Maintenance of PTGS requires the production of abRNA [abRNA (low)] to generate secondary siRNAs. Transgene transcription might be associated with abRNA production because transgenes usually lack introns and are regulated by artificial regulatory elements. Artificial promoters might recruit incomplete transcription machinery (TM*). Alternatively, lack of introns might prevent the elimination of abRNA by the spliceosome. If RDR6 only used abRNA as a template, the accumulation of abRNA but not of steady-state mRNA, would be the most crucial step for maintaining PTGS. A portion of the primary dsRNA enters the nucleus to initiate nuclear RNAi. The trigger could be the dsRNA itself or the 24-nt siRNAs that are produced from nuclear dsRNA by DCL3. The targeting of coding regions by nuclear RNAi could affect the accuracy of Pol II, leading to frequent premature termination of transcription and thereby to enhanced generation of abRNA. Nuclear RNAi involves dsRNA amplification that requires Pol IV and RDR2. The resulting secondary nuclear dsRNA reinforces nuclear RNAi and probably provides the RNA component of a putative silencing signal. The signal RNA could be bound to a carrier protein, enabling long-distance movement of the signal throughout the plant. Unloading the signal RNA into accompanying cells initiates PTGS. The RNA could be either processed into siRNAs or directly loaded onto AGO1. Upon targeting the abRNA, secondary siRNAs would be synthesized to maintain PTGS. The 21-nt siRNAs move through the plasmodesmata into neighbouring cells. In a transgene-expressing cell, they associate with abRNA to produce secondary siRNA. As a result of this amplification step, the siRNA concentration would stay constant in transgenic cells surrounding the 'silencing inducer cell' (A, 1 to 9). In wild-type cells, no abRNA and no secondary siRNA would be produced. Thus, the siRNA concentration would decline with the distance to the 'silencing inducer cell' (B, 1 to 9). Likewise, unloaded signal RNAs cannot mediate secondary siRNA production. RdDM is only initiated in the 'silencing inducer cell'. However, RdDM can be efficiently established in transgenic plants that have the potential to undergo spontaneous silencing (S-PTGS). This might indicate that in S-PTGS plants, a second abRNA threshold was reached [abRNA (high)]. A high abRNA concentration would be required to initiate RDR6-mediated secondary dsRNA production (broken arrow). The dsRNA would enter the nucleus to induce nuclear RNAi. Because nuclear RNAi is essential for generating the signal RNA, only S-PTGS-competent cells could re-initiate silencing signal production.

with respect to recruitment of transcription complexes. Incomplete assembly of transcription complexes, as can occur by using non-plant regulatory elements, could enhance abRNA production (Figure 1, 'TM*' versus 'TM'). In any case, accumulation of abRNA seems to be essential for maintaining PTGS. Mature mRNA molecules are targeted and cleaved but secondary dsRNA production and transitive silencing seems to be inefficient. Thus, it is reasonable to assume that secondary dsRNA is only generated from abRNA templates (Figure 1). In other words, the AGO1–siRNA complex targets both mRNA and abRNA but RDR6 will be recruited only in the case of abRNA. One can speculate that secondary siRNA production proceeds independently of any DCL activity given that RDR6 directly produces small RNAs. *N. crassa* QDE-1 (NcRDR6) performs two different reactions on single-stranded RNA (ssRNA) templates, which supports this idea. Either extensive RNA chains forming template-length

duplexes or ~9–21-nt RNA molecules are produced *in vivo* [27].

RDR6-mediated silencing of endogenous genes

AtRDR6 is also involved in the biogenesis of 'endogenous siRNAs' in *Arabidopsis*, which are genetically defined at specific loci [47–49]. Like transgene-derived siRNAs, these *trans*-acting siRNAs (tasiRNAs) target transcripts for cleavage in a process that involves AGO1 (TAS1 and presumably TAS2) or AGO7 (TAS3) [47,50,51]. tasiRNAs arise by phased, DCL4-processing of dsRNA formed by AtRDR6 activity on RNA polymerase II transcripts [35,48,49,52]. Functional redundancy exists among the four plant DCLs [51,52], thus, tasiRNAs might also be processed by DCL2 and DCL3. However, these alternatively processed tasiRNAs can have relatively low or no cleavage activity on their targets [49,52]. tasiRNAs mediate the cleavage of endogenous gene transcripts that are important in the transition from a juvenile to an adult phase of vegetative development before flowering [52,53]. These data show that the RDR6 is not only involved in transgene silencing and virus resistance but also plays an important role in natural plant development.

Nuclear RDR activity

In addition to cytoplasmic PTGS processes, transgene-mediated gene silencing, as well as virus and viroid infections, can be associated with nuclear RNAi, including RNA-directed DNA methylation (RdDM) and heterochromatin formation in plants [39,44,54,55]. RdDM is based on the presence of nuclear dsRNA. It seems likely that primary dsRNA and secondary cytoplasmic dsRNA triggers nuclear RNAi given that it is produced during S-PTGS (Figure 1). Primary cytoplasmic dsRNA might, for example, originate from viral RNA/RNA replication intermediates. Importantly, we suggest that siRNA-priming of abRNA would not result in stable secondary dsRNA production but would lead to direct generation of secondary siRNAs. This would be implemented either directly through RDR6 or in an indirect process by complete DCL-mediated cleavage of RDR6-produced secondary dsRNA. It is conceivable that siRNA-primed abRNA recruits a complex consisting of RDR6 and DCL4, which would lead to immediate cleavage of the dsRNA produced and would not allow the release of 'free dsRNA' that could enter the nucleus.

In the nucleus, the dsRNA is processed into 24-nt siRNAs, probably by DCL3 [53,56], and potentially also into 21-nt siRNAs by another nuclear DCL homologue. Plant DCLs have partially redundant functions, which makes it difficult to assign the four plant DCLs to the generation of either the 21-nt or the 24-nt siRNAs [53]. The 24-nt siRNAs are assumed to target homologous regions for *de novo* DNA and histone H3 lysine 9 methylation [57,58] (Figure 1, 'Transgenic plant nucleus'). Importantly, the nuclear AtRDR2 appears to be indispensable for RdDM in *Arabidopsis* [57], indicating that the production of nuclear secondary dsRNA is essential. However, the function of nuclear siRNAs in this process is not fully understood. Upon hybridization to complementary nascent transcripts, the 24-nt siRNAs might enable RDR2-mediated secondary dsRNA production. In plants, a fourth DNA-dependent RNA polymerase, Pol IV, was recently found to be essential for nuclear RNAi [59,60]. Pol IV was suggested to be involved in the production of secondary dsRNAs by directly transcribing methylated DNA. The enzyme could be guided by the 24-nt siRNAs to the target DNA. Pol IV transcripts could then serve as a template for the RDR2 to generate the secondary dsRNA. The secondary dsRNA, in turn, is required to enable maintenance of RdDM and histone modification [58].

There is indirect evidence that nuclear RNAi contributes to PTGS [17]. It was suggested that dense methylation of coding regions could provoke premature termination of primary transcription, leading to increased production of abRNA that would reinforce PTGS [36] (Figure 1). However, in this context, in contrast to transgene silencing, targeting of endogenous coding regions is not (or only poorly) associated with RdDM of the corresponding endogene [44]. Protection of endogenous coding regions against methylation could be based on low accessibility of nascent transcripts for Pol IV and/or RDR2, which would affect secondary nuclear dsRNA synthesis. Nascent transcripts of endogenes could be shielded by components of the spliceosome or the transcription machinery against hybridization of siRNAs and/or against binding of a putative RdDM complex (Figure 1, 'Wild-type nucleus'). As a consequence, nuclear dsRNA would not be amplified, which would result in the absence or inefficient *de novo* methylation and heterochromatin formation of endogenous targets. However, endogenous genes, for example transposons, retroelements and 5S rDNA sequences appear to be silenced by nuclear RNAi [13,61], indicating that nuclear RNAi contributes to natural gene regulation at individual loci. At present, it is not clear why some endogenes are targets for nuclear RNAi whereas others are not.

As in plants, nuclear RNAi also mediates heterochromatin assembly in *S. pombe*. The RNA-induced initiator of transcriptional gene silencing (RITS) effector complex guides siRNAs to homologous DNA to initiate heterochromatin formation [62–64]. This mechanism requires the single *S. pombe* RNA-directed RNA polymerase Rdp1 (SpRDR2/6). Rdr1 (SpRDR2/6) executes its function as a component of the RNA-directed RNA polymerase complex (RDRC), which contains two highly conserved proteins, the putative RNA helicase Hrr1 and Cid12, a member of the polyA polymerase/2′-5′oligoadenylate synthetase family of proteins. The RNA template-dependent RNA polymerase activity of RDRC requires siRNAs and association with RITS. RDRC–RITS assembly and association with target sequences is, in turn, dependent on Dicer (Dcr1) and the Clr4 histone H3 lysine 9 methyltransferase [65].

Involvement of RDRs in long-distance spreading of silencing

In addition to limited and extensive cell-to-cell movement of silencing, PTGS can be associated with long-distance spreading of silencing [35–39,45,46]. Himber and coworkers [38] proposed a model in which a putative

silencing signal, that either contains or consists of 24-nt siRNA, moves throughout the plant vascular system (Figure 1). In the silencing signal-receiving cells, the production of secondary siRNA would be initiated by the priming of abRNA. 24-nt siRNAs are produced only in the nucleus of 'silencing inducer cells' but not in 'siRNA receiver cells', which shows that 'silencing signal receiver cells' are not capable of re-initiating the process that leads to the production of the putative silencing signal. The 24-nt siRNAs are probably retained in the phloem or companion cells. Thus, 24-nt siRNA-mediated generation of 21-nt siRNAs can only occur in or near the vasculature. Frank Schwach and co-workers [66] reported that in *Nicotiana benthamiana*, RdR6 (NbRDR6) is required for the cell to respond to the silencing signal, but not to produce or translocate it. This finding is consistent with the view that the RNA component of the signal would only initiate RDR6-mediated generation of secondary siRNA by priming abRNA. We suggest that silencing signal production requires a second threshold of abRNA to be exceeded [Figure 1, abRNA (high)] because it would occur in transgenic plants displaying spontaneous PTGS [46,67]. In case of a high abRNA concentration, the RDR6 would mediate un-primed synthesis of 'free dsRNA' that is capable of entering the nucleus where it could initiate the generation of the RNA component of the silencing signal.

The above hypothesis of long-distance spreading of silencing appears to be plausible and comprehensive. However, two studies on movement of silencing signals have revealed no correlation between the accumulation of 24-nt siRNAs and long-distance spreading of silencing [68,69]. Thus, RNA molecules other than siRNAs should be considered as components of the putative silencing signal. A virus-encoded suppressor of long-distance spreading of silencing (2b) is localized to the nucleus and interferes with *de novo* DNA methylation [70], indicating that the RNA silencing signal is produced in the nucleus. As suggested above, secondary siRNA synthesis might not be associated with the production of dsRNA that can be translocated into the nucleus. Thus, nuclear RNAi will be initiated in neither 'siRNA receiver cells' nor in 'silencing signal receiver cells', which would explain the deficiency in producing a silencing signal.

Control of virus accumulation

In *N. tabacum* and *Arabidopsis RDR1* knockout plant lines, sensitivity against infection with various viruses was increased [5,14]. The virus RNA accumulated to significantly higher levels and disease symptoms appeared to be more severe than in wild-type plants. In addition, a potato virus X strain that did not spread in wild-type tobacco became systemic in the NtRDR1-deficient line [5]. Although it is not clear how virus accumulation is controlled, one can speculate that RDR1 preferentially uses the RNA of certain viruses as a template to generate siRNA-like molecules. Loading the siRNA-like RNAs onto an AGO protein might then trigger virus RNA cleavage. This hypothesis is consistent with the observation that in the *Arabidopsis AtRDR1* mutant, the turnover of viral RNAs occurred at a substantially lower rate than in wild-type plants [14].

RDR6 has also been shown to play a role in mediating virus resistance [18,66,71,72]. RDR6-deficient *Arabidopsis* plants that were infected with various viruses showed an enhanced susceptibility to cucumber mosaic virus (CMV) only [18,71]. CMV, in contrast to other RNA viruses, might produce a putative aberrant RNA species that could serve as template for RDR6 to produce secondary dsRNA. Schwach and co-workers [66] found that in *N. benthamiana*, RDR6 is involved in defence against potato virus X (PVX) at the level of systemic spreading and in excluding the virus from the meristem. RDR6 appeared to use the RNA component of the silencing signal to produce secondary siRNAs. This implies that the spread of the silencing signal and the generation of secondary siRNAs precede systemic PVX infection. In other words, in new developing leaf and meristem cells, the RNA silencing mechanism becomes activated before virus invasion. The hypothesis was recently supported when RDR6-deficient *N. benthamiana* plants were infected with various viruses at different growth temperatures [72]. At 21°C, RDR6-deficient plants showed enhanced susceptibility to PVX only compared with wild-type plants. However, at 27°C, much higher levels of turnip crinkle virus (TCV) and tobacco mosaic virus (TMV) accumulated in RDR6-deficient plants but not in wild-type plants. High levels of virus accumulation correlated with the development of severe symptoms and were, at least for TMV, associated with invasion of the meristem. Feng Qu and co-workers [72] have confirmed previous data demonstrating that the RNA-mediated silencing mechanism of plants is involved in virus defence and that this mechanism is enhanced at higher temperatures [73]. In addition, they showed that the temperature-dependence was correlated with RDR6 activity.

Concluding remarks

RDRs play an important role in different cellular processes. We are just beginning to understand the complexity of how these enzymes are implicated in the regulation of gene expression. They are involved in transcriptional, post-transcriptional and translational processes and act in the cytoplasm as well as in the nucleus. Extensive genetic and biochemical analysis will be necessary to further elucidate the mechanistic function and the protein structures of this class of RNA-producing enzymes. A focus should be placed on the characterization of the substrate specificity and on the identification of components that associate with RDRs.

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Supplementary data

Supplementary data associated with this article can be found at doi:10.1016/j.tplants.2006.01.003

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